GERMINATION, REGENERATION AND PIGMENT DETECTION
IN *Nelumbo nucifera* Gaertn.
(PINK ASIAN LOTUS)

NORAINI BINTI MAHMAD

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR
2012
GERMINATION, REGENERATION AND PIGMENT DETECTION
IN *Nelumbo nucifera* Gaertn.
(PINK ASIAN LOTUS)

NORAINI BINTI MAHMAD

DISSERTATION SUBMITTED IN FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR
2012
UNIVERSITY OF MALAYA
ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **NORAINI BINTI MAHMAD** (I.C/Passport No: **770124-03-5960**)
Registration/Matric No: **SGR 090012**
Name of Degree: **MASTER OF SCIENCE (MSc.)**


**GERMINATION, REGENERATION AND PIGMENT DETECTION IN**

*Nelumbo nucifera* Gaertn. (PINK ASIAN LOTUS)

Field of Study: **PLANT BIOTECHNOLOGY**

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya (“UM”), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate’s Signature                                      Date **12/09/2012**

Subscribed and solemnly declared before,

Witness’s Signature                                      Date **12/09/2012**
Name: **ROSNA MAT TAHA**
Designation: **PROFESOR**
ABSTRACT

In vivo and in vitro germination of three types of seeds including matured (black), immature or fruits (green) and young (yellow) of Nelumbo nucifera Gaertn. (pink Asian lotus) were done. The black and green seeds both gave the highest responses to technique of scarified with medium-sand paper and germinated in liquid (tap water) and solid (MS basal media) substrates. The black seeds with germination rate of 67.01±0.28% in vivo and 78.35±0.61% in vitro, while, green seed with 75.12±0.16% in vivo and 100.00% in vitro germination. The highest shoot length in seeds without cotyledon for black and green seed with 28.00±0.55 mm and 26.00±0.16 mm, respectively. The highest shoot length was in media with pH 5.5 for both black seed and green seeds and with 64.03±0.02 mm and 32.55±0.04 mm, respectively. The maximum root length for both black and green seeds was 14 mm on the 14th day with 3 green shoots and 6 white primary roots. Whilst, maximum root length for yellow seeds were on the 18th day with 3 green shoots and 6 white primary roots. For storage purposes, the optimum concentration for the formation of encapsulation matrix was 3.0% sodium alginate (NaC₆H₇O₆). Encapsulated explants were soaked in 100 mM calcium chloride dehydrate (CaCl₂·2H₂O) solution for 30 minutes. Through sodium alginate encapsulated method (4±1°C), seeds germination reduced from 100% (day 1) to 53.3±1.2% (day 90), while, through frozen whole seeds method (-20±1°C), 100% germination rate until 60 days in storage. After 90 days in storage, the germination rate was still high with 93.3±0.6%.

Direct regeneration of Nelumbo nucifera Gaertn. were successfully achieved from green seed explants cultured on solid MS media supplemented with combinations of 1.5 mg/l BAP and 0.5 mg/l NAA with 10.33 ±0.23 shoots per explant (true-to-type), and with 3.67 ±0.31 roots per explant. Direct regeneration of Nelumbo nucifera Gaertn.
were successfully achieved from yellow seed explants cultured on solid MS media supplemented with combinations of 0.5 mg/l BAP and 1.5 mg/l NAA with 16.00±0.30 shoots per explant, with new characteristics of layered multiple shoots. Roots were formed on solid MS basal media. Some formation of abnormal shoots (pinkish, red and oval leaf) occurred from green seed explants on solid MS media supplemented with combinations of 1.0 mg/l BAP and 2.5 mg/l NAA, 2.5 mg/l BAP and 2.5 mg/l NAA, 1.5 mg/l BAP and 2.0 mg/l NAA and 1.5 mg/l BAP and 2.5 mg/l NAA. Furthermore, the highest shoots per explant for green seed explants was in 8.8mg/l MS powder (double strength) with mean 19.03±0.05 shoots per explant. The highest shoots per explants for yellow seed explants was on 4.4 mg/l MS powder (full strength) with mean 16.06±0.06 shoots per explant. Media with pH 5.5 resulted in the highest height of shoots for green and yellow seed explants with mean 12.04±0.7 mm and 16.03±0.30 mm, respectively. While, the highest height of shoots for green seed explants with 9.41±1.11 mm in 250.00 mm light distance. The highest height of shoots with 16.67±0.23 mm for yellow seed explants in 200.00 mm light distance. Solid MS basal media was optimum for root formation within 4 weeks for green seed explants and after 24 weeks for yellow seed explants on solid MS media supplemented with 0.5mg/l BAP and 1.5mg/l NAA. In double layer media, the highest number of shoots per explant was both in ratio liquid to solid 2:1 with mean 16.67±0.23 number of shoots per explant with formation of primary and secondary roots for explants from yellow seeds with formation of layered multiple shoots, while mean 9.00±0.15 number of shoots per explant for green seeds.

Extraction from lotus stamen was analyzed through HPLC system. Pigment detected was found to have the total carotenoid content of 526.96 ± 0.52 µg/g DW, whereas for individual carotenoid β-carotene (460 ± 10.28 µg/g DW) was found with a relatively high concentration and neoxanthin (39.26 ± 0.82 µg/g DW) was found in lower
concentrations. One unknown carotenoid also was detected. For coating experiments, at room temperature with acidic conditions (pH 1-6), yellow pigment changed to the darker colour, yellow-brown, while, at strong alkaline condition (pH 12-14), the colour change to yellow-green. The optimum yellow colour was at pH 8-10. The highest glossiness was in 1.0ml/30.0ml v/v of pigment-resin solution with mean of 74.67±0.33°. Yellow pigment in 20% PMMA (resin) was as stable as pigment-resin added with 1% tartaric acid, coated on glass slides. In contrast, the addition of 1% citric acid reduced the carotenoid color. Extraction for antimicrobial activity from in vivo and in vitro rolled leaves showed no inhibition zone to all tested bacteria Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus cereus and E.coli. However, the highest inhibition zone was from in vivo sample for both on fungi, Fusarium sp. and Trichoderma sp. with mean 9.0±0.1 mm and with 7.3±02 mm diameters, respectively. Inhibition zone for in vitro samples were 8 times lower than in vivo with mean of 2.0±0.4 mm on Fusarium sp. and 1.1±0.3 mm on Trichoderma sp.

Soil samples collected from Tasik Chini with mean pH of 3.97±0.02, while the optimum survival for acclimatization plantlets was on black clay loam (mean pH 6.03±0.29) that commercialized at all the nurseries in Malaysia. The most acidic soils were collected from Tasik Chini with the lowest survival rate (31.34±0.77%) for green seed and 11.11±0.51% for yellow seeds. The highest survival rate was in black clay loam for both plantlets from green and yellow seeds with 83.01±0.23% and 69.22±0.43%, respectively. The highest plantlets height was obtained from plant acclimatized under full sunlight exposure with 10 times from shady exposure. Acclimatized plantlets showed the same character as mother plants, even though at the beginning of transferred with layered multiple shoots (new character was formed in vitro only).
ABSTRAK

Percambahan tiga jenis biji benih *Nelumbo nucifera* Gaertn. (lotus Asia merah jambu) secara *in vivo* dan *in vitro*, termasuk biji benih matang (hitam), tidak matang atau buah (hijau) dan muda (kuning) telah dikaji. Respon terbaik untuk kedua-dua biji benih hitam dan hijau adalah melalui kaedah gosokkan menggunakan kertas pasir dan percambahan di dalam ceair (air paip) dan pepejal (MS tanpa hormone). Kadar percambahan bagi biji benih hitam adalah 67.01±0.28% *in vivo* dan 78.35±0.61% *in vitro*. Manakala, percambahan bagi biji benih hijau adalah 75.12±0.16% *in vivo* dan 100.00% *in vitro*. Panjang pucuk yang tertinggi adalah daripada biji benih tanpa kotiledon bagi kedua-dua biji benih hitam dan hijau dengan masing-masing 28.00±0.55 mm dan 26.00±0.16 mm. Panjang pucuk yang tertinggi adalah daripada media dengan pH 5.5 bagi kedua-dua biji benih hitam dan hijau dengan masing-masing 64.03±0.02 mm dan 32.55±0.04 mm. Panjang akar yang maksima bagi kedua-dua biji benih hitam dan hijau adalah 14 mm pada hari ke-14 dengan 3 pucuk hijau dan 6 akar primer yang putih. Manakala, panjang akar yang maksima bagi biji benih kuning adalah 17 mm pada hari ke-18 dengan 3 pucuk hijau dan 6 akar primer yang putih. Untuk tujuan penyimpanan, kepekatan optima untuk pembentukan matriks pengkapsulan ialah 3.0% sodium alginate (NaC₆H₇O₆). Eksplan yang dikapsulkan direndam di dalam larutan 100 mM kalsium klorida dehidrat (CaCl₂.2H₂O) selama 30 minit. Melalui kaedah pengkapsulan sodium alginate (4±1°C), percambahan biji benih menurun daripada 100% (hari pertama) kepada 53.3±1.2% (hari ke-90). Manakala, melalui kaedah pembekukan biji benih asal (-20±1°C), 100% kadar percambahan sehingga 60 hari tempoh penyimpanan. Selepas 90 hari penyimpanan, kadar percambahan masih tinggi dengan 93.3±0.6%.

Regenerasi *Nelumbo nucifera* Gaertn secara langsung berjaya dicapai melalui eksplan daripada biji benih hijau yang dikultur di atas media MS pepejal yang ditambah dengan
kombinasi 1.5 mg/l BAP dan 0.5 mg/l NAA dengan 10.33 ±0.23 pucuk per eksplan (sama seperti induk), dan lengkap dengan 3.67±0.31 akar per eksplan. Regenerasi Nelumbo nucifera Gaertn secara langsung berjaya dicapai melalui eksplan daripada biji benih kuning yang dikultur di atas media MS pepejal yang ditambah dengan kombinasi 0.5 mg/l BAP dan 1.5 mg/l NAA dengan 16.00±0.30 pucuk per eksplan, dengan ciri yang iaitu pucuk berlapis-lapis. Akar terbentuk di dalam media MS tanpa hormone. Beberapa pembentukan pucuk-pucuk tidak normal (merah jambu, merah dan bujur) daripada eksplan biji benih hijau di dalam media MS pepejal yang ditambah dengan kombinasi 1.0 mg/l BAP dan 2.5 mg/l NAA, 2.5 mg/l BAP dan 2.5 mg/l NAA, 1.5 mg/l BAP dan 2.0 mg/l NAA serta 1.5 mg/l BAP dan 2.5 mg/l NAA. Tambahan lagi, pucuk per eksplan yang tertinggi adalah daripada biji benih hijau yang dibekalkan dengan 8.8 mg/l serbuk MS (dua kali ganda kekuatan) dengan purata 19.03±0.05 pucuk per eksplan. Pucuk per eksplan tertinggi adalah daripada biji benih kuning dengan 4.4 mg/l serbuk MS (kekuatan penuh) dengan purata 16.06±0.06 pucuk per eksplan. Medium dengan pH 5.5 memberikan tinggi pucuk yang tertinggi bagi biji benih hijau dan kuning dengan purata 12.04±0.70mm dan 16.03±0.30mm, masing-masing. Manakala, tinggi pucuk yang tertinggi bagi biji benih hijau adalah 9.41±1.11 mm dalam 250.00 mm jarak dari cahaya. Tinggi pucuk yang tertinggi bagi biji benih kuning pula adalah 16.67±0.23 mm dalam 200 jarak dari cahaya. Media MS pepejal adalah optimum untuk pembentukan akar dalam tempoh 4 minggu bagi biji benih hijau dan kuning dan 24 minggu bagi biji benih kuning daripada media MS yang ditambah dengan 0.5 mg/l BAP and 1.5mg/l NAA. Bagi media dua lapis, pucuk per eksplan tertinggi adalah di dalam kadar 2:1 dengan purata 16.67±0.23 pucuk per eksplan dengan pembentukan akar primer dan sekunder untuk biji benih kuning dengan pucuk berlapis-lapis dan 9.00±0.15 pucuk per eksplan bagi biji benih hijau.
Pengekstrakan pigmen daripada stamen lotus telah dianalisa melalui sistem HPLC yang mengandungi jumlah karotenoid sebanyak 526.96 ± 0.52 µg/g DW di mana individu carotenoid terdiri daripada β-carotene (460 ± 10.28 µg/g DW) dengan kepekatan yang tinggi dan neoxanthin (39.26 ± 0.82 µg/g DW) dengan kepekatan yang rendah. Satu jenis carotenoid yang tidak dikenalpasti juga diperolehi. Untuk eksperimen cat, pada suhu bilik dengan keadaan berasid (pH 1-6) pigmen kuning bertukar kepada warna yang lebih gelap, kuning kecoklatan. Manakala, dalam keadaan alkali kuat (pH 12-14), warna bertukar kepada kuning kehijauan. Kuning yang optima adalah pada pH 8-10. Kilatan tertinggi adalah dalam 1.0ml/30.0ml v/v larutan pigmen-resin dengan purata 74.67±0.33°. Pigmen kuning dalam 20% PMMA (resin) adalah stabil dengan pertambahan 1% asid tartaric yang dicat pada kaca. Sebaliknya, pertambahan 1% asid sitrik akan memudarkan warna karotenoid. Pengekstrakan untuk mengkaji aktiviti antimikrobial ke atas bakteria menunjukkan tiada lingkaran zon perencatan. Sebaliknya, zon lingkaran perencatan wujud pada kedua-dua kulat, Fusarium sp. dan Trichoderma sp. dengan purata 9.0±0.1mm serta 7.3±02 mm diameter bagi sampel in vivo. Zon lingkaran perencatan untuk sampel in vitro adalah 8 kali lebih rendah berbanding sampel in vivo iaitu dengan purata 2.0±0.4mm pada Fusarium sp. dan 1.1±0.3mm pada Trichoderma sp.

Sampel tanah dari Tasik Chini menunjukkan purata pH 3.97±0.02, manakala kemandirian optima untuk plantlet aklimatisasi di dalam tanah liat hitam (purata pH 6.03±0.29) yang dikomersilkan di semua nurseri seluruh Malaysia. Tanah yang paling berasid diambil dari Tasik Chini adalah yang paling rendah kadar kemandiriannya, dengan 31.34±0.77% bagi biji benih hijau dan 11.11±0.51% bagi biji benih kuning. Kadar kemandirian yang tertinggi adalah di dalam tanah liat hitam bagi kedua-dua plantlet daripada biji benih hijau dan kuning dengan masing-masing 83.01±0.23% dan
69.22±0.43%. Planlet yang paling tinggi diperolehi daripada tumbuhan yang diaklimatisasikan di bawah sinaran penuh matahari dengan kadar 10 kali dibandingkan dengan yang berada di tempat teduh. Plantlet yang diaklimatisasi menunjukkan ciri-ciri yang sama dengan induk, walaupun pada mulanya biji benih kuning menunjukkan ciri-ciri pucuk berlapis-lapis (ciri-ciri baru hanya di dalam in vitro sahaja).
ACKNOWLEDGEMENT

I would like to express my greatest appreciation and thanks to my supervisor, Professor Dr. Hjh. Rosna Mat Taha for her guidances, contributions and encouragements to accomplish this research.

Special thanks to Institute of Biological Sciences (ISB), Faculty of Science, University Malaya and also to Institute of Research Management and Consultancy of University Malaya (IPPP), for the grant PS206/2009C and all the facilities provided.

Thank you very much to all members of Plant Tissue Culture Laboratory (B2.5); Azani, Azah, Ain, Anis, Ashikin, Imah, Ina, Kinah, Madiha, Shima, Sya and Zue for their sharing of invaluable knowledge in tissue culture, technical advices and encouragements during the research.

My deepest gratitude to my beloved parents, husband and children (Farah Hannani, Adi Ashraff and Muhammad Jazarie) for their constant encouragement and support throughout of my study.

Priceless.......ALHAMDULILLAH.......
# LIST OF CONTENTS

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>vi</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF CONTENTS</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xviii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xxv</td>
</tr>
</tbody>
</table>

## CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION OF *Nelumbo nucifera* Gaertn.  
1.1.1 Classification and Nomenclature of *Nelumbo sp.*  
1.1.2 Comparison of Characteristics between Nelumbonaceae (lotuslily) and Nymphaeae (waterlily)  
1.1.3 Adaptations of Nelumbonaceae

1.2 INTRODUCTION TO TISSUE CULTURE OF AQUATIC PLANTS

1.3 RESEARCH OBJECTIVES

## CHAPTER 2 SEED GERMINATION OF *Nelumbo nucifera* Gaertn.,  
*In Vivo* and *In Vitro*

2.1 EXPERIMENTAL AIMS

2.2 MATERIALS AND METHODS

2.2.1 Source of Seeds

2.2.2 Preparation for Breaking Seeds Dormancy

2.2.3 Preparation of *In Vivo* and *In Vitro* Germination (Solid and liquid MS Media)

2.2.3.1 Sterilization of whole Seeds

2.2.3.2 Preparation of Solid MS Media

2.2.4 *In Vitro* Germination on solid MS Media

2.2.4.1 Identification of the Best Seeds Germination

2.2.4.2 Identification of the Optimum pH

2.2.5 Encapsulation Matrix

2.2.5.1 Preparation of 3% (w/v) Sodium alginate Solution (NaC₆H₇O₆)

2.2.5.2 Preparation of Calcium Chloride Dehydrate Solution (CaCl₂·2H₂O)

2.2.5.3 Encapsulation Techniques and Bead Formation

2.2.5.4 Low Temperature Storage

2.2.6 Data Analysis

2.3 RESULTS
2.3.1 Identification of the Highest Seeds Germination, *In Vivo* (in tap water) and *In Vitro* (solid MS Basal Media (1962))

2.3.2 Identification of the Best Excised Method for Seed Germination

2.3.3 Identification of the Optimum pH for Seed Germination

2.3.4 Primary Root Induction Versus Time

2.3.5 Identification of Suitable Sodium Alginate Encapsulated Matrix

2.3.6 Identification of Effect of Low Temperature Storage on Seeds Germination

2.4 SUMMARY OF RESULTS

CHAPTER 3 REGENERATION THROUGH TISSUE CULTURE SYSTEM OF *Nelumbo nucifera* Gaertn.

3.1 EXPERIMENTAL AIMS

3.2 MATERIALS AND METHODS

3.2.1 Sterilization Protocol for Aseptic Seedlings

3.2.2 Preparation of *In Vitro* Explants

3.2.3 Preparation of Solid MS Basal Media (1962) with Hormones

3.2.4 Preparation of Hormones Stocks

3.2.5 Sterile Culture Conditions

3.2.6 Subculture

3.2.7 *In Vitro* Cultures of *Nelumbo nucifera* Gaertn.

3.2.7.1 Induction of Shoots and Roots Formation on Solid Media with Combinations of BAP and NAA

3.2.7.2 Identification of the Optimum MS Strength

3.2.7.3 Determination of the Optimum pH

3.2.7.4 Effects of Distance of the Light Source

3.2.7.5 Effects of Double-layered Liquid and Solid Media

3.2.8 Data Analysis

3.3 RESULTS

3.3.1 Shoot and Root Formation on Solid Media with Combinations of BAP and NAA

3.3.2 Determination of the Optimum MS Strength

3.3.3 Identification of the Optimum pH

3.3.4 Effects of Distance of the Light Source

3.3.5 Root Formation on Solid and Liquid media

3.3.6 Effects of Double-layered Liquid and Solid Media

3.4 SUMMARY OF RESULTS

CHAPTER 4 PIGMENT DETECTION AND EXTRACTION in *Nelumbo nucifera* Gaertn.
4.1 EXPERIMENTAL AIMS

4.2 MATERIALS AND METHODS
   4.2.1 Plant Sample Preparation
   4.2.2 Pigment Preparation
   4.2.3 Effects of pH on Carotenoid Stability
   4.2.4 Carotenoid Extraction and Analysis
      Through HPLC System
      4.2.4.1 Extraction of carotenoids
      4.2.4.2 Data Analysis
   4.2.5 Carotenoid as Natural Coating
      4.2.5.1 Resin Preparation and Coating
      4.2.5.2 Effect of Cobinder
      4.2.5.3 Data Analysis
   4.2.6 Chlorophyl Extraction and Microbial Activity
      4.2.6.1 In vivo plant samples
      4.2.6.2 In vitro plant samples
      4.2.6.3 Test Organisms
      4.2.6.4 Sample extraction
      4.2.6.5 Antimicrobial test
      (paper disk diffusion method)

4.3 RESULTS
   4.3.1 Effect of pH on Carotenoid Colour
   4.3.2 Analysis of total and individual carotenoid content in lotus flower stamen
   4.3.3 Coating from Lotus Stamen
   4.3.4 Lotus Leaves Extraction, in vivo and in vitro
   4.3.5 Inhibition Zone from Lotus Leaf Extracts, in vivo and in vitro

4.4 SUMMARY OF RESULTS

CHAPTER 5 ACCLIMATIZATION OF *Nelumbo nucifera* Gaertn.

5.1 EXPERIMENTAL AIMS

5.2 MATERIALS AND METHODS
   5.2.1 Source of In Vitro Regeneration
   5.2.2 Transplanting Plantlets to Containers
      5.2.2.1 Determination of Sterile Soil Type and pH on Survival Rates of Green Shoots and Yellow Shoots
      5.2.2.2 Effects of Sunlight Exposure on Stem Height and Leaf Width
      5.2.2.3 Growth Development of Plantlets
   5.2.3 Data Analysis

5.3 RESULTS
   5.3.1 Determination of Soil Types and pH on Survival Rates
LIST OF TABLES

Table 1.1: The difference physiology between Nelumbonaceae (lotuslily) and Nymphaeae (waterlily) 10
Table 1.2: Wide range of aquatic plant species tissue culture. 21
Table 1.3: Valuable madicinal parts of Nelumbo nucifera Gaertn. (Pink Asian lotus) 24
Table 1.4: Valuable parts of Nelumbo nucifera Gaertn. for medicinal purposes 27
Table 2.1: Responses of different seeds maturity to various of break seed dormancy in vivo (in tap water) and in vitro (on solid basal MS media with pH 5.5 and maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 4 weeks). 40
Table 2.2: Responses of different methods in culturing black, green and yellow seeds on solid basal MS media with pH 5.5 and maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 4 weeks. 43
Table 2.3: The effect of different pH in germination of seeds on solid MS basal media. Cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 4 weeks. 45
Table 2.4: The response of root induction on solid MS basal media. Cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 4 weeks. 47
Table 2.5: Effects of different concentration of sodium alginate (NaC₆H₇O₆) and calcium chloride dehydrate (CaCl₂·2H₂O) on bead formation. 51
Table 2.6: Effect of storage period on germination rate of Nelumbo nucifera Gaertn at 4±1°C (sodium alginate encapsulated) and -20±1°C (frozen whole seeds) in 90 days. 54
Table 3.1: The response on different combination concentration of BAP and NAA on juvenile shoots from green and yellow seeds, cultured on solid MS media at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 24 weeks. 68
Table 3.2: In vitro development of shoots in different strength of MS media. Cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 4 weeks. 77
Table 3.3: The effect of different pH in regeneration of shoots. Cultures were maintained on MS media supplemented with growth regulators (BAP+NAA) at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 4 weeks. 79
Table 3.4: The effect of light distance in regeneration of shoots. Cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 4 weeks. 80
Table 3.5: The effect of root induction on solid and liquid media. Cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 4 weeks. 81
Table 3.6: The effect of solid level in regeneration of shoots. Cultures were maintained at 25±1°C with 16 hours light
and 8 hours dark, with 1000 lux intensity of light for 4 weeks.

Table 4.1: Functions of carotenoids

Table 4.1: Effects of pH on yellow pigment (carotenoid) from lotus stamen

Table 4.2: Total and individual carotenoid content (µg/g DW) of stamen tissue of lotus flower from Tasik Chini in Pahang, Malaysia.

Table 4.3: Extraction ratio of dried lotus stamen to 70% acetone solution

Table 4.4: The glossiness and ratio of pigment and resin in coating process

Table 4.5: Antimicrobial activity of hydroethanolic extracts from rolled leaves of *Nelumbo nucifera* Gaertn., in vivo and in vitro

Table 5.1: Effects of soil pH on survival of acclimatized plantlets from green seed and yellow seed

Table 5.2: Growth of stems and leaves on different age

Table 5.3: Observation on lotus characteristics within 8 months acclimatization under full sunlight
LIST OF FIGURES

Figure 1.1: *Nelumbo nucifera* Gaertn. (sacred lotus or pink Asian lotus) in Chini Lake, Pahang, Malaysia 2

Figure 1.2: *Nelumbo lutea* (Willd) Pers. (yellow American lotus) in North America, United States of America 2

Figure 1.3: Nutrious parts of lotus rhizomes containing alkaloid and liensinine were highly demands as vegetable in Japan, South Korean and Asian (a)rhizome (b)nodal buds (c)edible lotus rhizome 7

Figure 1.4: Nutrious parts of lotus fruits containing containing alkaloid, liensinine, saponins, phenolics and carbohydrates (a)green fruit (b)matured seeds (c)dried lotus nut 7

Figure 1.5: Illustration by Swindells,P. (1983). Waterlilies. Timber Press, Portland, Oregon. (a)Lotuslily (b) waterlily 12

Figure 1.6: (a) Pink Asian lotus flower Nelumbonaceae (lotuslily), (b) Pink Nymphaea (waterlily) 13

Figure 1.7: Structure of *Nelumbo nucifera* Gaertn. matured seed (black seed) as the survival tactic for viability up to 200 years. Image by http://www.victoria-adventure.org/ 15

Figure 1.8: The swelling of the seed to almost double in volume within 2 months in water 16

Figure 1.9: The multipurpose of lotus parts as nutrious and medicinal vegetable. (a)flower (b)stamen (c) seed (d)fruit (e)leaf (f)nodal rhizome 25

Figure 2.1: General structure of a dicot seed of *Nelumbo nucifera* Gaertn. 30

Figure 2.2: General structure of rhizomes of *Nelumbo nucifera* Gaertn 31

Figure 2.3: The germination process of whole black seeds soaked in water for 2 months in 4±1°C (a) swollen moist-prechilling seed (b) cracked seed (c) shoot elongation (d) No root formation within 8-10 weeks 41

Figure 2.4: Three type of seed sources germinated on solid MS basal Medium with different techniques a) Black seed (mature) b) Green fruit (immature) (c) Yellow seed (young) (d) whole seed (e) Green seeds with one white cotyledon (f) yellow seeds without cotyledon turn to green within 6 days (g)whole seed with white inner layer (h)white cotyledon turn to green within 3 days (i) shoots elongation of yellow
seed with green cotyledon

Figure 2.5: Shoots elongation from half cotyledon with pH 5.5.

Figure 2.6: The Root standard growth of *Nelumbo nucifera* Gaertn.

Figure 2.7: Roots formation started after 10-24 weeks in culture depends on the age of the seeds (a) 6-months-old on solid MS basal media (b) White Primarily roots (c) Roots elongation

Figure 2.8: Sodium alginate encapsulated shoots with different soaking period in calcium chloride dehydrate (a) 10 minutes; clear, soft and no definite shape (b) 20 minutes; clear, solid and round bead (c) 30 minutes; cloudy, solid and round bead

Figure 2.9: Sodium alginate encapsulated shoots storage in 4±1°C (a)sodium alginate solution (b) encapsulated shoots (beading using syringe) were soaked in calcium chloride for 30 minutes (c) bead placed on MS basal media (d) Beads cracked (e) Healthier shoot elongation after 15-45 days in storage (f) shoot elongated after 90 days in storage

Figure 3.1: (a) Effects of BAP and NAA on shoot formation from the juvenile shoots (from green and yellow seeds) of *N. Nucifera* after 24 weeks in culture on MS medium. The cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 24 weeks.

Figure 3.1: (b) Effects of BAP and NAA on shoot formation from the juvenile shoots (from green seeds) of *N. Nucifera* after 24 weeks in culture on MS medium. The cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 24 weeks.

Figure 3.1: (c) Effects of BAP and NAA on root formation from the juvenile shoots (from green seeds) of *N. Nucifera* after 24 weeks in culture on MS medium. The cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 24 weeks.

Figure 3.2: Tissue culture from green seed explants on MS solid media supplemented with 1.5 mg/l BAP and 0.5 mg/l NAA, 16 hrs light and 8 hrs dark, at 25±1°C (a) Week 2, shoots elongation (b) Week 12, first rolled leaf(c) Week 16, first unrolled leaf
Figure 3.3: Tissue culture from yellow seed explants on MS solid media supplemented with 0.5 mg/l BAP and 1.5 mg/l NAA, 16 hrs light and 8 hrs dark, at 25±1°C (a) Week 2, shoots elongation (b) Week 12, first rolled leaf (c) Week 16, first unrolled leaf (d) Week 24, layered shoots without roots formation.

Figure 3.4: Abnormal leaf cultured on MS media supplemented with 1.5 mg/l BAP and 0.5 mg/l NAA after 10 weeks in culture (a) Pink Shoots (b) rolled red rolled leaf (c) Green spongy tissue (d) Green and round leaf (e) Pink and green oval leaf (f) Red and green oval leaf (g) Dark green stem (h) Stem with tiny thorns (prickles)

Figure 3.5: Rooting of coatless green seed explants on solid MS basal media (a) Dark green rolled plumul (b) After 24 weeks (6 months), explants with the same characteristic of rolled plumul but additional with 5 white primarily roots (pink at the root tips).

Figure 3.6: Subculture of 2-week-old green seed explants on solid MS media supplemented with 1.5mg/l BAP and 0.5mg/l NAA (a) 4-week-old; shoots elongation (b) 16-week-old; 3 rolled leaf and 1 unrolled leaf (c) 24-week-old; primarily roots formation (d) Internode characteristic; 2 pink and 2 white primarily roots

Figure 3.7: Subculture of 2-week-old yellow seed explants on solid MS media supplemented with 0.5mg/l BAP and 1.5mg/l NAA (a) 2-week-old yellow seed explants (b) 4-week-old; shoots elongated (c) 6-week-old; layered shoots (d) 12-week-old; layered shoots elongation (e) 14-week-old; rolled leaf (f) 24-week-old; primarily roots formation

Figure 3.8: Effects of solid (with combination BAP and NAA) and liquid (MS basal) media on juvenile explants (a) Shoots without root elongated on solid MS media and need to subculture or transfer every 21 days to avoid necrosis (b) Shoots without root elongated on solid MS media added with liquid MS basal
(c) Plantlet maintained in double layer media (without transferring for up to 10 months) (d) Rooting from yellow seeds with shoots elongation on solid MS media supplemented with 0.5 mg/l BAP and 1.5 mg/l NAA (base) and liquid MS basal media (top)

Figure 3.9: Regeneration of yellow seed explants (a) 10-month-old vigorous shoots elongation in double layer media (without root) (b) The best explants (juvenile shoot excised from yellow seed) regenerated on optimum solid MS media supplemented with 0.5 mg/l BAP and 1.5 mg/l NAA for 20 weeks, developed with new characteristics (layered multiple shoots). Then, transferred 4 weeks to MS solid media for roots formation. The cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 24 weeks.

Figure 4.1: Source of carotenoids in lotus (a) fresh yellow stamen of lotus collected from Tasik Chini, Pahang (b) dried ground yellow stamen (c) yellow stained on cotton (d) dried grey residue (e) Bright and clear yellow supernatant in acetone solution

Figure 4.2: Yellow pigment change to yellow-brown with range pH 1-6. Original at pH 8-10. While, range pH 12-14 with yellow-green colour

Figure 4.3: HPLC analysis of individual carotenoid of lotus flower. Chromatography, absorption and retention time of lotus stamen carotenoid content and composition (6.2 min – neoxanthin, 26.6 min – unknown carotenoid, 28.1 min – beta-carotene)

Figure 4.4: Effects of 1% tartaric acid and 1% citric acid as the stabilizer of coating

Figure 4.5: 24 hours after coated on slide (a) Pigment in acetone solution (b) Pigment+PMMA(resin) (c) Pigment+PMMA(resin)+1% tartaric acid (d) Pigment+PMMA(resin)+1% citric acid

Figure 4.6 (a) 10cm diameters in vivo lotus rolled leaves Ilmu, University of Malaya and (b) green 70% v/v ethanol extraction, in vivo (c) 6-month-old of in vitro rolled leaves with 1cm diametre (d) dark green 70% v/v ethanol extraction, in vitro

Figure 4.7: Antifungal activity of hydroethanolic extracts
of *Nelumbo nucifera* Gaertn. higher from *in vivo* rolled leaves compared to *in vitro* (a) *Fusarium* sp.; mean 9.0±0.1mm (b) *Trichoderma* sp.; mean 7.3±0.2mm

Figure 4.8 : Zero response of antibacterial activity of hydroethanolic extracts from rolled leaves of *Nelumbo nucifera* Gaertn., *in vivo* and *in vitro* (a) *Staphylococcus aureus* (b) *Pseudomonas aeruginosa* (c) *Bacillus cereus* (d) *E.coli*

Figure 5.1 : 8-month-old plantlets in double layer media (solid:MS+0.5mg/l BAP+1.5mg/l NAA, liquid:MS basal media) from yellow seed explants. (a) cloudy solid agarose MS media and clear liquid MS media (b) clumps of multiple shoots and roots; washed with tap water (c) layer multiple shoots (new characteristic; differ from mother plants)

Figure 5.2 : 8-month-old plantlets solid MS media supplemented with 1.5mg/l BAP 0.5mg/l NAA, from green seed explants. (a) multiple shoots (b) true-to-type creeper plantlets with horizontal stem and rolled leaves (c) internode; runner with new roots

Figure 5.3 : Acclimatized plantlets in clay loam with ratio 2:1, (solid to liquid) for adaptation to real environment. (a) 14th days uncovered plantlets in culture room (25±1°C) for initial adaptation. Plant transferred to greenhouse after 21 days. (b) 6-month-old in greenhouse (with 4 unrolled leaves and fibrous roots)

Figure 5.4 : Comparison of acclimatized lotus in stem height under full sunlight and shady exposure at 30±1°C

Figure 5.5 : Effect of sunlight exposure to growth of lotus plants in clay loam with ratio 2:1, solid to liquid. (a) after 8 months acclimatized under full sunlight exposure with 12 leaves and 4 rotten leaves. b) after 8 months acclimatized under shady greenhouse with 2 leaves.

Figure 5.6 : First 4 leaves of 8-month-old acclimatized plantlets, emerged from water surface (a) adaxial of leaves: red spotted (b) abaxial of leaves: waxy green (c) stem: hollow and prickles

Figure 5.7 : Acclimatization in fresh water aquarium with black clay loam soil and associate with guppy fish. (a) 8-month-old plantlets (b) shoots elongation (c) after 8 months acclimatization

Figure 5.8 : 8 months acclimatization in containers a) floating leaves b) first 3 leaves (emerged), b) 1 rolled leaf and 1 petiol

Figure 5.9 : Lotus characteristics and adaptation within 8 months acclimatization under full sunlight in clay loam soil (mean pH 6.03±0.29)
LIST OF ABBREVIATIONS

BAP
Benzyaminopurine

CaCl₂·2H₂O
Calcium chloride dehydrate

2,4-D
2,4- Dichlorophenoxyacetic acid

HCL
Hydrochloric acid

IAA
Indole-3-acetic acid

IBA
Indolebutyric acid

2-iP
2-isopentenylaminopurine

Kinetin
6-furfurylamino purine

kPa
Kilo Pasca

mg/l
Milligram per liter

min
minute

MS
Murashige and Skoog

MgCO₃
Magnesium carbonate

NAA
Naphthalene acetic acid

NaOH
Sodium hydroxide

NaC₆H₇O₆
Sodium alginate

Rpm
Rotation per minute

Tween 20
Polyoxyethylene sorbitan monolaurate

v/v
Volume per volume

w/v
Weight per volume